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<p>(21) International Application Number: PCT/US88/02601 (22) International Filing Date: 29 July 1988 (29.07.88)  (31) Priority Application Number: 080,479 (32) Priority Date: 31 July 1987 (31.07.87) (33) Priority Country: US  (71) Applicant: THE BOARD OF TRUSTEES OF THE LE- LAND STANFORD JUNIOR UNIVERSITY [US/ US]; Stanford, CA 94035 (US).  (72) Inventors: BURG, Lawrence, James; 648 Pegasus Lane, Foster City, CA 94404 (US). POULETTY, Philippe, Jacques; 100 San Mateo Drive, Menlo Park, CA 94025 (US). BOOTHROYD, John, Charles; 42 Roosevelt Circle, Palo Alto, CA 94306 (US).</p>		<p>(74) Agent: NEELEY, Richard, L.; Leydig, Voit &amp; Mayer, 350 Cambridge Avenue, Suite 200, Palo Alto, CA 94306 (US).  (81) Designated States: AU, DK, FI, JP, KR, NO.  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: SELECTIVE AMPLIFICATION OF TARGET POLYNUCLEOTIDE SEQUENCES</p> <p>(57) Abstract</p> <p>A method is provided for multiplying the number of copies of a target polynucleotide sequence comprising a series of primer hybridization, extending, and denaturing steps to provide an intermediate double-stranded DNA molecule containing a promoter sequence (through the use of a promoter-sequence-containing primer) incorporated upstream from the target sequence. The double-stranded DNA intermediate is then used to grow multiple RNA copies of the target sequence. The resulting RNA copies can be used as target sequences to produce further copies. Multiple cycles of this sort can thereby exponentially increase the number of target sequence copies.</p> <div data-bbox="850 1360 1321 1478"> </div>		

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<p>(54) Title: SELECTIVE AMPLIFICATION OF TARGET POLYNUCLEOTIDE SEQUENCES</p> <p>(57) Abstract</p> <p>A method is provided for multiplying the num- ber of copies of a target polynucleotide sequence com- prising a series of primer hybridization, extending, and denaturing steps to provide an intermediate double-stranded DNA molecule containing a promo- ter sequence (through the use of a promoter-sequence- containing primer) incorporated upstream from the target sequence. The double-stranded DNA interme- diate is then used to grow multiple RNA copies of the target sequence. The resulting RNA copies can be used as target sequences to produce further copies. Multiple cycles of this sort can thereby exponentially increase the number of target sequence copies.</p> <div data-bbox="846 1356 1317 1461"> </div>		

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## SELECTIVE AMPLIFICATION OF TARGET POLYNUCLEOTIDE SEQUENCES

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### FIELD OF THE INVENTION

This invention is related to diagnostic assays in which the presence of a particular gene is being detected, whether for detection of the gene itself or  
10 detection of the organism containing the gene, and is particularly directed to techniques in which the number of copies of the gene being detected is enzymatically increased prior to the detection process. It is further related to any process requiring the generation of many  
15 copies of a specific polynucleotide sequence.

### BACKGROUND OF THE INVENTION

A number of diagnostic assays have been developed which rely on detection of the presence of a particular DNA or RNA sequence as an indication of the  
20 presence of an analyte, e.g., a bacterium, virus, or genetic defect, in a sample. In some cases the diagnostic gene is present in sufficient quantities to be detected directly, whether by hybridization, reaction  
25 with a specific antibody, or by some other method. However, if the gene of interest is present in a small amount or the background caused by similar sequences present in the sample is sufficiently high, reliable and sensitive detection of the targetted gene is difficult.  
30 An ambiguous result is not satisfactory in a diagnostic test.

Various techniques for increasing the sensitivity and specificity of such diagnostic procedures have been developed. Amplification of the target by  
35 cell culture, an efficient but time consuming technique, has been for long the only reliable method. Other techniques increase sensitivity of the detection

system using sensitive reporter groups attached to the probe which will combine with the target. Examples of sensitive reporter groups would include radioactive and fluorescent molecules. Enzymes, such as peroxidase or alkaline phosphatase coupled to the probe, also improve sensitivity through their catalytic action on substrate chromophores. Increased sensitivity may also be obtained by an amplification of the reporter groups. Such amplification has been achieved through avidin-biotin interactions, networking with nucleic acids, or the direct enzymatic replication of an RNA reporter group. This latter technique generates up to 1,000,000 copies of the RNA in about 12 minutes. Another technique amplifies the target nucleic acid sequence rather than the reporter groups used in the detection system.

One method for amplification of target nucleic acid is known as the polymerase chain reaction or PCR technique and has been developed for detecting the genes responsible for genetic defects. This method uses specific oligonucleotide primers in repeated cycles of target DNA denaturation, primer annealing, and extension with a DNA polymerase. Extension products generated from one primer serve as additional target sequences for the other primer. The degree of amplification of a target sequence is controlled by the number of cycles that are performed and is theoretically calculated by the simple formula  $2^n$  where  $n$  is the number of cycles. Given that the average efficiency per cycle ranges from about 65% to 85%, 25 cycles are needed to yield from 0.3 to 4.8 million copies of the target sequence.

Although the polymerase chain reaction is a very sensitive and promising method, there are some limitations and disadvantages inherent in this technique. For example, each cycle of polymerase chain reaction provides at best only a 2-fold amplification, and thus a high number of cycles (between 20 and 30) is

required to achieve substantial amplification. Furthermore, the high-temperature denaturation that occurs in each PCR cycle typically inactivates the enzyme used and thus requires repeated addition of expensive enzyme.

5 Accordingly, techniques that increase the rate of gene amplification (thereby requiring less enzyme and fewer cycles) would be highly advantageous to all diagnostic techniques that involve the detection of a specific target nucleotide sequence and any other pro-  
10 cedure needing an increased number of specifically amplified polynucleotides (RNA or DNA).

#### RELEVANT LITERATURE

The PCR method is described in a number of  
15 publications, including Saiki et al., "Enzymatic amplification of beta-globin genomic sequences and restriction site analysis of sickle cell anemia", Science (1985) 230:1350-1354; Saiki et al., "Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA  
20 with allele-specific oligonucleotide probes", Nature (1986) 324:163-166; and Scharf et al., "Direct cloning and sequence analysis of enzymatically amplified genomic sequences", Science (1986) 233:1076-1078. Also see European Patent Application publication no. 0 200 362  
25 A2 (Application No. 86302298-4), published December 10, 1986, which claims priority to three U.S. patent applications: Serial No. 716,975, filed March 28, 1985; Serial No. 791,308, filed October 10, 1985; and Serial No. 828,144, filed February 7, 1986.

30

#### SUMMARY OF THE INVENTION

The present invention provides a method for rapidly multiplying (through an enzymatic cycle) the number of copies of a target polynucleotide sequence by  
35 alternating two procedures that make copies from a target template. In a first series of steps, an intermediate double-stranded polynucleotide is produced com-

prising a promoter followed by the target sequence. The double-stranded intermediate is then used in a second process to prepare multiple RNA copies using an RNA polymerase that binds to the promoter region of the double-stranded intermediate. Each of the RNA copies can then be used as a target sequence to initiate another amplification cycle by preparing a second (or further) collection of double-stranded promoter-containing intermediates using reverse transcriptase. The process of the invention therefore provides a technique that multiplies in vitro the number of copies of a target polynucleotide sequence present in a sample much faster than prior processes. The method is applied in a specific embodiment to a diagnostic assay for Toxoplasma gondii.

#### BRIEF DESCRIPTION OF THE DRAWING

The present invention will be better understood by reference to the following detailed description of the invention when considered in combination with the drawing that forms part of the specification, wherein:

The Figure is a schematic diagram showing polynucleotides present at different stages of the method of the invention.

#### DESCRIPTION OF SPECIFIC EMBODIMENTS

The present invention provides a method for multiplying the number of copies of a target polynucleotide sequence and therefore is particularly useful in diagnostic assays intended to recognize specific polynucleotide sequences that are present in small amounts in a sample. It is also useful in any method benefiting from rapid generation of polynucleotides of specific sequence.



The target polynucleotide sequence may be only a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not  
5 necessary that the target sequence to be amplified be present initially in a pure form. It can be a minor fraction of a complex mixture, such as a portion of a nucleic acid sequence due to a particular microorganism, which organism constitutes only a minor fraction  
10 of a particular biological sample being analyzed.

The starting reaction mixture containing polynucleotides can contain more than one target sequence if desired. Therefore, the present process is useful not only for producing large amounts of one specific  
15 polynucleotide target sequence but also for amplifying simultaneously more than one different target sequence located on the same or different polynucleotide molecules. If more than one target sequence is present, the only modification required of the present invention  
20 is to provide primers (discussed below) for each of the desired target sequences.

Any specific polynucleotide target sequence can be amplified by the present process. It is only necessary that a sufficient number of nucleotides at  
25 both ends of the sequence be known in sufficient detail so that two oligonucleotide primers can be prepared as described below. The greater the knowledge about the bases at both ends of the sequence, the greater can be the specificity of the primers for the target sequence, and thus the greater the efficiency of the process. It  
30 will be understood that the word primer as used herein can refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding the terminal sequence or sequences of the  
35 target to be amplified. For example, when a nucleic acid sequence is being inferred from a known protein sequence, a collection of primers containing sequences

representing all possible codon variations based on degeneracy of the genetic code will be used for each strand. One primer from this collection will be homologous with the end of the target sequence.

5           The method begins by preparing a double-stranded polynucleotide intermediate that contains the target sequence and additionally contains a promoter located upstream from the target sequence. This double-stranded intermediate is prepared by a series of steps  
10 using short oligonucleotides as primer sequences and extending the primers using the longer polynucleotide strand to which the primer binds as the template. The complementary target strand is obtained in a single-stranded state (if not already in that form) and is hybridized to an oligonucleotide sequence containing a  
15 promoter sequence upstream from a binding sequence complementary to a region in the complementary strand at or near the 3' end of the target region in the complementary strand. The binding sequence in this primer is  
20 substantially equivalent to a sequence in the target polynucleotide molecule found 5' to the specific target sequence being copied or at the 5' end of the target sequence. This primer is used to initiate the synthesis of a DNA molecule, using a DNA polymerase (such as  
25 reverse transcriptase), of the same sense as the original target strand.

A second oligonucleotide is then hybridized to this newly synthesized strand. This second oligonucleotide primer is complementary to a region corresponding to the 3'-end of the target molecule. The  
30 number of nucleotides between the primer regions is preferably less than 300, more preferably less than 200, but more than 10, more preferably more than 15. When the second primer is extended, a copy of the target sequence and the first primer are formed. The  
35 resulting product, therefore, contains the promoter region 5' to the target sequence that is the object of this first part of the method of the invention.

The intermediate promoter-containing double-stranded polynucleotide is then used as a template for a DNA-dependent RNA polymerase capable of binding to the promoter region that has been created in the intermediate. The polymerase enzyme copies the target sequence, thereby providing multiple RNA copies of the target sequence downstream from the promoter. The number of copies produced depends on the promoter, the RNA polymerase used, and the reaction conditions, but 10 copies are readily produced and 100 or more copies can be prepared by selecting strong promoters, active RNA polymerases, and suitable reaction conditions.

Each of the RNA copies can be used as a template for the production of additional copies of the specific target. Reaction with the second oligonucleotide used above, extension of the primer to give the complementary sequence, reaction with the first primer and extension of the resulting hybrid in both directions (the first primer acting as primer for production of the target copy and the 3' terminus of the complementary strand acting as primer so that a copy of the promoter region is made) will produce a similar double-stranded promoter-containing intermediate described above that was used as a template for preparing RNA copies. RNA-producing cycles utilizing the promoter dependent RNA polymerase can then be repeated. If only 10 RNA copies are produced per cycle, three cycles will produce a one thousand-fold increase in the number of target sequences present in the reaction medium. If 100 copies of RNA are produced per template, one million copies of the target sequence will be produced by three cycles.

The method as described above assumes the presence of either a double-stranded polynucleotide target comprising a target strand and a complementary strand to the target strand or the presence of a complementary target strand if the initial target is a single-stranded

polynucleotide, such as RNA. If the target is initially single-stranded RNA or DNA, the complementary target strand can be prepared in a manner similar to that described above using an oligonucleotide primer. For example, the second oligonucleotide primer described above is complementary to a region corresponding to the 3'-end of the target molecule. This second oligonucleotide primer, or a different primer complementary to a different region 3' to the target sequence, can be used to prepare the complementary strand used in the first step of the procedure. Another variation is to start with the oligonucleotide primer that is complementary to the target (rather than its complementary) strand. The promoter-sequence-containing primer is then hybridized to the complementary strand that is extended from the first primer.

The oligonucleotide primers are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands. The primer sequence therefore need not reflect the exact sequence of the template to which it binds. For example, a non-complementary nucleotide fragment may be attached to the 5'-end of the primer, with the remainder of the primer sequence being complementary to the template strand. Of course, as described above, one such non-complementary nucleotide fragment will be a promoter sequence required by the invention. However, even when a promoter sequence is not present, other non-complementary nucleotide fragments may be attached. For example, a sequence providing a restriction endonuclease cleavage site can be provided to allow preparation of amplified target sequences ready for insertion into a plasmid. Alternatively, non-complementary bases can be interspersed in the binding sequence of the primer, provided that the primer sequence has sufficient com-

plementarity with the sequence of the strand to be amplified (or its complementary strand) to hybridize therewith and form a template for synthesis of the extension product. For example, a single nucleotide can  
5 be replaced in a primer of moderate length (e.g., about 15 nucleotides) in order to provide a specific restriction endonuclease cleavage site.

If desired promoter sequences can be included in both primers to further multiply the number of copies  
10 produced by a complete cycle.

The operation of the method of the invention can be readily seen by reference to the Figure and the following more detailed description. Standard nomenclature and orientation are used for the schematic single- and double-stranded polynucleotides shown in the  
15 Figure and in the following discussion. The left end of the upper strand of double-stranded polynucleotides is the 5' end with the right end of the same strand being the 3' end (indicated by arrowheads). Since complementary strands have opposite orientations, the bottom strand is shown with its 3' end to the left and its  
20 5' end to the right. In order to avoid confusion, the bottom strand is shown in this orientation even when not shown as part of a double-stranded polynucleotide. The same convention is followed for sequences set forth  
25 in the text (including the claims) in which the various sequences are identified by letters and numbers. Not all the products of each stage of the process are shown; only those relevant to this invention are illustrated.

In the first line of the Figure can be seen a  
30 double-stranded target polynucleotide molecule comprising a series of regions defined in terms of the operations that will be carried out on this molecule during later stages. As discussed previously, a double-stranded target is made for the initial step if the original  
35 target is a single-stranded RNA (or DNA) molecule. At the 5' end of the polynucleotide is a segment identi-

fied as X1 that will not be copied in later operations. This is followed by a segment of sequence P1 that comprises a part of the sequence of a primer used in a later stage. Sequence P1 is followed by target sequence  
5 T, which is the sequence being copied. A sequence P2, which binds with a different primer, is present at the 3' end of T. Additional nucleotides may be present past P2 but are not copied. These nucleotides are designated X2 in the figure. Neither X1 nor X2 are re-  
10 quired for the operation of the present invention and are therefore optionally present, ranging in number from zero to many hundreds or even thousands of nucleotides. The apostrophe or "prime" designation indicates the complementary sequence present on the opposite  
15 strand. This double-stranded target molecule is denatured in the presence of an oligonucleotide primer and then conditions changed to allow annealing of the oligonucleotide and target as shown in line 2.

Line 2 of the Figure shows a first oligonucle-  
20 otide primer, PR-P1, of complex structure but including the sequence P1 at its 3'-end, hybridized to the P1' segment of the denatured target molecule. The 5'-portion of this oligonucleotide primer, PR, contains a sequence which could, in its double-stranded form, com-  
25 prise a functional promoter for an RNA polymerase. Sequence PR may contain additional nucleotides without adversely affecting the invention. The PR-P1 primer is extended by an appropriate enzyme (either a DNA-dependant DNA polymerase for a DNA target or a RNA-dependant  
30 DNA polymerase for a RNA and/or DNA target) to provide a strand, PR-P1-T-P2-X2 as shown in line 3 of the Figure. The single-stranded nature of the promoter region is designated in the Figure by the absence of contact between the two strands at this region and in the text  
35 in the following manner:

PR — P1--T--P2--X2  
 X1'-P1'-T'-P2'-X2'.

5 This (and other) primer of the invention is preferably relatively short, namely 50 or fewer nucleotides, in order to allow rapid hybridization (annealing) and cycling, especially with automatic equipment.

10 After denaturing the double-stranded polynucleotide shown in line 3 of the Figure, a second primer of sequence P2' is added to the mixture under annealing conditions as shown in line 4. Sequence P2' is equivalent to the sequence P2' previously identified and is itself complementary to the P2 segment of PR-P1-T-P2-X2.

15 Line 5 of the Figure shows the product obtained by extending the primer sequence P2' on the template shown. The promoter region at this stage is now double-stranded and therefore can be used for binding to a DNA-dependent RNA polymerase. In the text, this double-stranded intermediate is written:

20 PR--P1--T--P2--X2  
 PR'-P1'-T'-P2'

The copies produced by the RNA polymerase are shown in line 6 of the Figure. The copied segments, all downstream from the promoter region, are designated P1<sup>R</sup>-T<sup>R</sup>-P2<sup>R</sup> (the superscript R denotes an RNA copy).  
 25 This RNA molecule can be the same as or slightly longer or shorter than P1-T-P2 since it will contain all of sequence PR-P1 at and after the initial nucleotide that is copied by the RNA polymerase. If the original target strand is a single-stranded DNA target, P1<sup>R</sup>-T<sup>R</sup>-P2<sup>R</sup>  
 30 is the RNA equivalent of the original DNA plus any portion of the promoter-containing segment PR copied by the RNA polymerase or minus any nucleotides not copied by the polymerase.

35 If the RNA copies are to be recycled for additional amplification, the steps previously discussed for preparation of the promoter-containing double-stranded DNA intermediate are repeated. It is not

necessary to use the same primer segments P1 and P2' or the same promoter segment PR, although PR-P1 and P2' are already available and can be used without modification. If other primer sequences are used, the resulting copies will be slightly shorter or larger than the first copies, but this does not adversely affect the invention unless there is some particular need or desire to retain the original P1-T-P2 sequence. Initial annealing with a P2' primer followed by extension of the primer provides the product shown in line 7 of the figure. Denaturation of the double-stranded intermediate and annealing with the primer PR-P1 provides the product shown in line 8. This intermediate can be extended in both directions since the P1 sequence acts as a primer using the complementary strand as a template and the complementary strand acts as a primer using sequence PR as a template.

If the same primers are used in this step as were used in the steps indicated by lines 1 through 5, then the product will be similar to that shown in line 5, differing only in the extent of the top strand. This will not affect any subsequent RNA polymerizations as only the lower, template strand is used by the RNA polymerase. If different primer sequences are used the resulting template strand may be larger or shorter depending on the primers used. Provided that at least one of the primers used in this step has, at its 5'-end, a sequence representing a promoter for an RNA polymerase, the resulting material will still be capable of further amplification. Accordingly, the promoter-containing double-stranded DNA intermediate of line 9 in the figure can be used with DNA-dependent RNA polymerase to again produce multiple RNA copies.

When the initially used primers hybridize with unintended sequences in the DNA mixture containing the target molecule, advantages can be achieved by using different primers when the cycle is repeated. For ex-



ample, if a particular target nucleotide is being detected in a crude analyte containing many polynucleotide sequences, such as when a bacterial or parasitic infection is being detected in a human fluid or tissue sample, hybridization of the primer oligonucleotides with host DNA can result in amplification of unintended host sequences. Since a promoter sequence will be present in the amplified background material, a significant increase in the amount of a specific background sequence is seen along with the increase in the target sequence. In order to avoid this problem, different primers and/or promoters can be used during different cycles to prevent amplification of the background. For example, a different binding region ( $P1^X$  and/or  $P2^X$ ) that falls within the target sequence near or overlapping the binding region of the original primer can be used in the second cycle. Although there may be binding with other background polynucleotide sequences whose amplification is not desired, it is unlikely that the same background material would be amplified. The primers used in sequential steps can be described as being a nested set of primers, if this embodiment of the invention is followed, since successive pairs of primers would be found closer together on the complementary strands of the target molecule. The second (and successive) cycles can also use a different promoter region (e.g.,  $PR^X$ ) that binds a different RNA polymerase. Degradation of the first RNA polymerase remaining in solution followed by introduction of the second polymerase that binds to  $PR^X$  will multiply the target copies but not the background copies, although an additional group of background copies may arise. Additionally, primers from earlier cycles, which may be present in excess, can be removed if desired. Suitable techniques for removing primers rely on characteristics that distinguish them from the target, such as size or the existence of the primers as single-stranded DNA

when the target is present as double-stranded DNA or as RNA. For example, gel chromatography will remove small primers from larger targets while specific nucleases or antibodies will remove primers from double-stranded targets.

As an example of use of either the nested set of primers or different primers with different promoters, assume that in a first amplification cycle the desired target sequence is multiplied by a factor of fifty. At the same time, binding of primers to a background sequence occurs so that the background sequence is also multiplied by a factor of fifty. If the second amplification cycle uses nested primers and a fifty-fold multiplication again occurs, the target sequence will be multiplied by 2500 while (at worst) there will be two background sequences each multiplied by fifty. After three cycles of nested primers, the multiplication factor for target will be 125,000 with (at worst) three background sequences multiplied by fifty.

The individual steps of the method of the present invention are all conventional and can be carried out using known reagents and techniques. The only specially designed reagents are the primers PR-P1 and P2'. However, these segments can readily be obtained, either by total synthesis or isolation of the desired sequences from natural sources. Total synthesis is most readily practiced if the sequences P2 (and accordingly its complementary sequence P2') and P1 are known in the target molecule. Total synthesis of DNA segments containing promoter sequences is readily accomplished since promoter sequences themselves have been published. If the sequence of the target molecule is not known, the target molecule can be segmented by restriction endonucleases or other processes and segments selected for use in the invention. The RNA polymerase and its associated promoter sequence can be selected from the numerous available sources of these components. For

example, T7 RNA polymerase from the T7 bacteriophage is available from New England Biolabs. Other RNA polymerases include SP6 from the bacteriophage SP6, available from New England Biolabs, or K-12 from E. coli strain K-12, which is available from Sigma Chemical Company, St. Louis, Missouri. The corresponding promoters can be isolated from the organism from which the polymerase is obtained or synthesized chemically, where the promoter sequence is known.

Other biochemical reagents used in the method of the invention include an enzyme capable of extending primer sequences to form double-stranded polynucleotides. Such enzymes include reverse transcriptase and other DNA polymerases. Particularly preferred enzymes are AMV reverse transcriptase from LifeScience Inc. or polymerase I (Klenow fragment) from Bethesda Research Laboratories, Pharmacia, U.S. Biochemicals or Biolabs.

The individual steps of the present invention are readily adaptable to automation. Reactions can be carried out in a single container by sequentially adding reagents and changing conditions as necessary. Denaturing conditions generally involve increased temperatures, particularly temperatures in the range from 95°C to 100°C. Annealing and primer extension occur at lower temperatures, typically 35°C to 50°C. Since many proteins are denatured along with the polynucleotides at the higher temperatures indicated, additional proteins may be added as necessary. Other reagents present can include buffers to maintain the reaction at the appropriate pH and ionic conditions as well as mononucleotide triphosphates (eventually labeled or modified to provide a detectable signal) to be used in the extension reactions.

If high temperatures are used to denature double-stranded polynucleotides, there is no requirement of separating reagents or solutions. Other denaturing conditions, such as use of solvents, are less

desirable because of the added separation steps when going from annealing to denaturing conditions and back to annealing conditions. However, numerous denaturing, annealing, and extending conditions are known and can be used if desired.

The multiple copies of the target sequence can be used in numerous ways. For example, the method of the invention can be used to prepare multiple copies of a gene for insertion into a plasmid or other target of a genetic engineering process. If used in a diagnostic assay for the target sequence, the detection step (hybridizing with a probe, reacting with an antibody or a specific ligand) can be carried out without isolating the amplified target from the reaction medium if the probe, antibody or ligand target is within the sequence T, i.e., does not encompass part of the primer or promoter regions. A probe target within the regions P1 and P2 can be selected, but separation of the target gene copies ( $P1-T-P2$  or  $P1^R-T^R-P2^R$ ) would be required in order to avoid binding with or interference by primer molecules. Labeled riboside triphosphates (e.g., radioactive labels, biotin or avidin labels) can also be used in steps in which RNA is produced. Additional uses of the polynucleotide products generated by this invention would include mutagenesis, genetic engineering and cloning, genetic analysis, therapy (both gene- and chemo- therapy), the production of protein (both in vitro translation and introduction into cells), and any other processes benefitting from multiple copies of specific polynucleotide sequences. For example, RNA regulatory molecules can be produced in large amounts.

The invention now being generally described, the same will be better understood by reference to the following detailed examples which are provided for purposes of illustration only and are not intended to be limiting of the invention.

EXAMPLE

The techniques of the invention were assessed using a cloned gene of Toxoplasma gondii, specifically the thirty-five-fold repetitive antigen gene identified as gene B1. The indicated gene was obtained from a recombinant DNA library containing inserts of genomic DNA from T. gondii (RH strain). The library was constructed in the expression vector lambda gt11 and was screened with antisera from an immunized rabbit. Recombinants thus identified were subcloned and further characterized. The target gene used in these studies was found to be part of a 2.2 kilobase-pair (kb) unit which is tandemly repeated many times in the genome of T. gondii. The identification and restriction map of this gene have been published (Boothroyd et al., "Antigen and Tubulin Genes of Toxoplasma Gondii", in Molecular Strategies of Parasitic Invasion, Ayabian et al., eds., UCLA Symposia Vol. 42, pp. 237-250, Alan Liss, New York, 1987). The nucleotide sequence of one complete repeat has been determined (but not published). The portion of the sequence relevant to the present work is reproduced below. The numbering system used measures from the EcoRI site defining the repeat in the genome. The sequence is shown as a double stranded molecule with the top strand being in the 5'-3' direction, left to right. The oligonucleotide segments are underlined in the strand of the same sense and thus the same sequence.

30 721  
AAAAAATGTG GGAATGAAAG AGACGCTAAT GTGTTTGCAT AGGTTGCAGT CACTGACGAG  
TTTTTTACAC CCTTACTTTC TCTGCGATTA CACAAACGTA TCCAACGTCA GTGACTGCTC  
OLIGO #0 OLIGO #1

781  
CTCCCTCTG CTGGCGAAAA GTGAAATTCA TGAGTATCTG TGCAACTTTG GTGTATTCCG  
35 GAGGGGAGAC GACCGCTTTT CACTTTAAGT ACTCATAGAC ACGTTGAAAC CACATAAGCG  
OLIGO #2

841  
AGATTGGTCG CCTGCAATCG ATAGTTGACC ACGAACGCTT TAAAGAACAG GAGAAGAAGA  
TCTAACCAGC GGACGTTAGC TATCAACTGG TGCTTCCGAA ATTCTTGTG CTCTTCTTCT

## OLIGO #3

5           Since the ultimate goal of the gene amplifica-  
tion was for potential use of the method in a diagnos-  
tic assay, the target gene was tested to see if it fit  
the remaining criteria necessary of a target gene for  
diagnosis. These are that the target gene be present  
10 in most or all strains of the parasite, ~~not present~~ in  
the genomes of other infectious agents (particularly  
those which might be confused in the diagnosis), and  
not detectable in the genome of the host. Preliminary  
investigations show that the gene is present in all  
15 strains of the parasite tested and that there is no  
cross-reactivity with the genomes of other organisms  
tested, including that of the human host.

A number of primers were evaluated for use in  
the method of the invention. These primers are identi-  
20 fied by underlining in the sequence set forth above.  
The primers were 20-40 nucleotides (including the op-  
tional promoter sequence) in length showing homology  
with the target sequence for efficient annealing. A  
guanosine (G) or cytosine (C) residue was present at  
25 the 3' end of each primer to assure effective base  
pairing from which extension would occur. Primers were  
selected with no more than 150 base pairs between pri-  
mers complementary to opposite strands so that exten-  
sion would be efficient and rapid. The primers were  
30 further selected to lack the ability to form stable  
base-pair structures with other primers or within a  
single primer which would prohibit a primer from an-  
nealing to the target sequence. Although not essential  
to the multiplication steps, the primers were chosen to  
35 flank convenient restriction sites to facilitate clon-  
ing and construction of probes for the "internal" am-

plified DNA (i.e., a probe specific for the amplified product but which would not hybridize to the primers). The "internal" amplified DNA is equivalent to section T in the general description and claims.

5           The reaction conditions used for generation of the double stranded fragment including the T7 RNA polymerase promoter were similar to those previously described (Saiki et al., Nature (1986) 324:163-166). The reaction medium contained in a 100  $\mu$ l volume, 10 mM  
10   Tris pH 7.5, 10 mM  $MgCl_2$ , 1.5 mM dNTPs, 1.5  $\mu$ M oligo-nucleotide primers, and variable amounts of target containing DNA depending on the experiment (from 0.15 pg to 1.0  $\mu$ g). Each cycle consisted of denaturation at  
15   90°C for 2 min (except for the first cycle, where denaturation occurred for 10 min), a quick chill on dry ice for 5 sec, a spin (to eliminate condensation at the top of the tube), annealing of primers at 35°C for 2 min, addition of two units of Klenow fragments of E. coli  
polymerase I, and extension at 35°C for 2 min.

20           The method of the invention was further carried out by including a promoter for the RNA polymerase of bacteriophage T7 at the 5' end of one of the target gene-specific oligonucleotides (oligo #1 identified above). The T7 promoter sequence is TAATACGACTCACTATAGGG.  
25   These additional 20 nucleotides were incorporated into the amplified double-stranded DNA products of the initial steps to provide products that are 20 bp larger than if oligonucleotides lacking this sequence were used. For example, the amplified DNA using oligos #1  
30   and #2 is 117 bp, 20 bp larger than the 97 bp of the target including homology to the oligonucleotides (and similarly with oligos #1 and #3, the product was 151 bp - 131 bp + 20 bp).

35           These added-on sequences functioned as an efficient promoter for T7 RNA polymerase. The in vitro T7 transcription reactions were performed according to instructions of the supplier of the T7 RNA polymerase.

The DNA template was taken directly from the reactions described above (which incorporated the promoter region) with no further treatment. An autoradiogram of the in vitro generated T7 RNA transcripts confirmed  
5 that they were 17 nucleotides shorter than the DNA template. In other words, the double-stranded DNA intermediate product of 151 base pairs gave a 134 nt RNA, 131 nt from target sequences and 3 nt (GGG) from the T7 promoter sequence. By incorporating [32P]-UTP  
10 into the RNA transcript, an approximately 3000-fold increase in the sensitivity of detecting amplified product was obtained as compared to ethidium bromide staining.

The assay of the invention was specifically  
15 designed to give high-specific-activity, low-yield RNA and therefore does not represent a maximum molar increase of target sequences generated from T7 transcription. However, it is known that under high-yield conditions, T7 RNA polymerase will produce between 50 and  
20 100 RNA molecules per DNA template molecule (Davanloo et al., "Cloning and expression of the gene for the bacteriophage T7 RNA polymerase", Proc. Natl. Acad. Sci. USA (1984) 81:2035-2039).

The procedure was carried out using several  
25 cycles of the T7RT amplification shown in the Figure and described above. This procedure is referred to here as the T7RT technique for convenience. This process has several advantages over the prior art PCR method. First, the target sequence to be amplified can  
30 be either RNA or DNA, which is important if the selected gene is highly expressed, since AMV reverse transcriptase can use either RNA or single-stranded DNA as a template. This is also relevant for applications in which the target sequences are exclusively RNA, as for  
35 retrovirus detection. Second, amplification from each full cycle, including both sequences, should be higher than with the PCR technique (up to 100 per cycle for



T7RT compared with a maximum of 2 per cycle for the PCR technique) thus allowing a reduced number of cycles (3 cycles for a  $10^6$ -fold amplification for T7RT versus at least 20-25 for PCR) and the corresponding use of a smaller amount of enzyme. Third, large amounts of RNA can be produced for use in any process when RNA is preferable to DNA such as translation into protein, chemical lability, etc.

Initial investigations of the T7RT method utilized the same oligonucleotides and target sequences as described in the previous sections. As indicated in the Figure, two enzymes are used sequentially for each cycle: AMV reverse transcriptase and T7 RNA polymerase. Repeated steps of denaturation, annealing, and extension are realized by changes of sample temperature (90-100°C for denaturation, 37-41°C for annealing) and addition of enzyme to a sample containing nucleic acid to be amplified, adequate buffer (indicated by enzyme supplies), NTPs and dNTPs, molar excess of oligonucleotide primers, and an inhibitor of RNase.

In a first experiment, the gene fragment initially synthesized to contain a promoter sequence (as described above) was used to demonstrate the feasibility of the T7RT procedure. First, the substrate DNA was transcribed by T7 RNA polymerase, which was expected to yield an RNA product of 134 nt. This material was then converted back to a double stranded DNA as described and shown in the Figure using AMV reverse transcriptase in the presence of radio-labeled dNTPs. The major DNA product expected (and obtained) was also 134 nt, with a small amount of material of 151 nt present because of the continued presence of the input gene fragments. A control sample was similarly treated except that T7 polymerase was omitted. Following this reverse transcriptase step, a second cycle of direct amplification by AMV RT was performed on each sample. This resulted in the synthesis of substantial amounts

of the 151 nt product by synthesis of the 134 nt product using oligo #1 (which has the extra 17 nucleotides of T7 promoter at its 5' end) as the primer. As the 134 nt material was about 10-fold more abundant (after one T7RT cycle) than the 151 nt product of the controlled reaction, the T7 amplification corresponded to that obtained by about 4.5 cycles of the PCR method alone. This amplification was achieved without optimizing the T7RT procedure, so that greater improvements in efficiency and cost over the ~~PCR~~ method are expected.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for multiplying the number of copies of a target polynucleotide sequence in a reaction medium, which comprises:
- 5 (1) sequentially hybridizing a target single-stranded polynucleotide molecule with primer sequences followed by extending said primers and denaturing, wherein at least one of said primer contains a promoter sequence, to produce a double-stranded DNA intermediate having a promoter sequence upstream from a target sequence and (2) growing multiple RNA copies of said target sequence from said intermediate using an RNA polymerase capable of binding said promoter.
- 10 2. The method of Claim 1, further comprising (1) preparing a second collection of double-stranded DNA intermediates having a promoter sequence upstream from said target sequence from said RNA copies by hybridizing said RNA copies with primer sequences, extending, and denaturing, and (2) growing multiple RNA copies from said second collection of double-stranded DNA intermediates.
- 15 3. The method of Claim 1, wherein said extending comprises contacting said reaction medium with a reverse transcriptase enzyme.
- 20 4. The method of Claim 1, wherein said method comprises hybridizing the anti-sense strand of a double-stranded target molecule with a first primer comprising a promoter sequence at its 5' end and a binding sequence equivalent to a sequence of the target molecule sense strand 5' to the target sequence, extending a first complementary strand from said first primer using said anti-sense strand as a template, denaturing to form a first collection of single-stranded polynuc-
- 25 30 35

leotide intermediates, hybridizing said first collection with a second primer that binds 3' to said target sequence in said sense strand, extending a complementary strand from said second primer using said first complementary strand as a template to provide said double-stranded DNA intermediate, and contacting said reaction medium with an RNA polymerase capable of binding said promoter.

5. The method of Claim 1, which comprises:

(1) hybridizing a single-stranded polynucleotide molecule of sequence  $X1'-P1'-T'-P2'-X2'$  that is complementary to a target molecule of sequence  $X1-P1-T-P2-X2$  with a first primer of sequence PR-P1 to form

$$\begin{array}{c} \text{PR} \text{---} \text{P1} \\ X1'-P1'-T'-P2'-X2'; \end{array}$$

(2) extending a complementary strand to form

$$\begin{array}{c} \text{PR} \\ P1--T--P2--X2 \\ X1'-P1'-T'-P2'-X2', \end{array}$$

followed by denaturing to form a first collection of single-stranded polynucleotides;

(3) hybridizing said first collection of single-stranded polynucleotides with a second primer of sequence P2' to form

$$\begin{array}{c} \text{PR-P1-T-P2-X2} \\ P2'; \end{array}$$

(4) extending a complementary strand to form

$$\begin{array}{c} \text{PR--P1--T--P2--X2} \\ \text{PR'-P1'-T'-P2'} \end{array}; \text{ and}$$

(5) growing multiple RNA copies of  $P1-T-P2$  having the formula  $P1^R-T^R-P2^R$  using an RNA polymerase capable of binding double-stranded promoter region

$$\begin{array}{c} \text{PR} \\ \text{PR'} \end{array};$$

wherein:

T is a target sequence;

T' is a sequence complementary to T;

25

PR-P1 is a first oligonucleotide primer  
in which PR comprises a promoter sequence and P1 is the  
binding sequence of said first primer;

5 P1' is a sequence complementary to P1;  
PR' is a sequence complementary to PR;  
P2' is a second oligonucleotide primer;  
P2 is a sequence in the target polynucle-  
otide complementary to P2';

10 X1 and X2 are sequences outside the P1-T-  
P2 sequence of said target molecule and individually  
may be present or absent;

X1' is a sequence complementary to X1;  
X2' is a sequence complementary to X2; and  
P1R-TR-P2R is an RNA molecule in which  
15 P1R is a sequence equivalent to P1, TR is a sequence  
equivalent to T, and P2R is a sequence equivalent to  
P2.

6. The method of Claim 5, wherein said method  
20 further comprises the steps of:

(6) hybridizing said multiple RNA copies of  
formula P1R-TR-P2R with said second primer to form  
P1R-TR-P2R  
P2';

25 (7) extending a complementary strand to form  
P1R-TR-P2R  
P1'-T'-P2',

followed by denaturing to form a third collection of  
single-stranded polynucleotides;

30 (8) hybridizing said third collection of  
single-stranded polynucleotides with said first primer  
to form

PR-P1  
P1'-T'-P2';

35 (9) extending complementary strands to form  
PR--P1--T--P2  
PR'-P1'-T'-P2'

(10) repeating step (5); and  
(11) optionally repeating steps (6)-(10);  
wherein in each cycle of steps (6)-(10), each  
PR-P1 and P2' oligonucleotide primer represents the  
5 corresponding primer from a preceeding cycle of steps  
or a primer different from the corresponding primer.

7. The method of Claim 5, wherein said target  
single-stranded polynucleotide molecule is RNA.

10 8. The method of Claim 5, wherein said target  
single-stranded polynucleotide molecule is DNA.

9. The method of Claim 5, wherein extending com-  
15 prises contacting said sequences to be extended with a  
reverse transcriptase.

10. The method of Claim 5, wherein said RNA poly-  
merase is T7 RNA polymerase.

20 11. The method of Claim 1, wherein said primers  
comprise binding sequences of from 10 to 30 nucleotides  
in length.

25 12. The method of Claim 1, wherein said primers  
are separated by less than 200 base pairs.

13. The method of Claim 1, wherein said promoter  
sequence is at the 5' terminus of a primer.

30 14. The method of Claim 1, wherein said target  
single-stranded polynucleotide molecule is DNA, extend-  
ing is accomplished using reverse transcriptase, and  
said primers are present in said intermediate no more  
35 than 150 base pairs apart.

27

15. A method of detecting a target polynucleotide sequence in a sample, which comprises:

    multiplying the number of copies of said target sequence according to Claim 1; and

5       detecting the presence of said multiplied copies.

10

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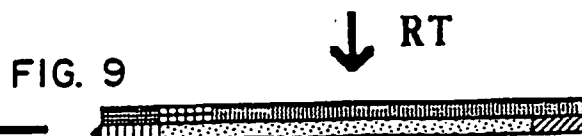
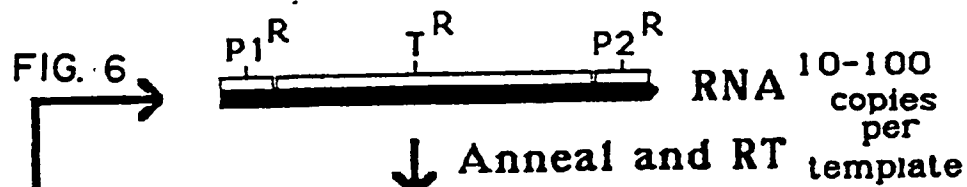
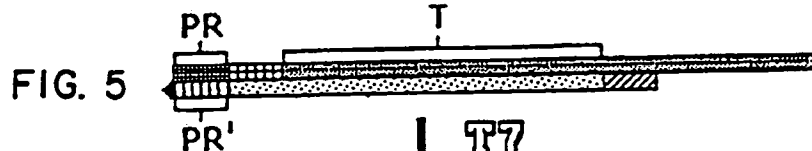
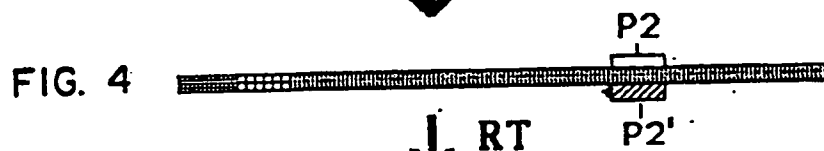
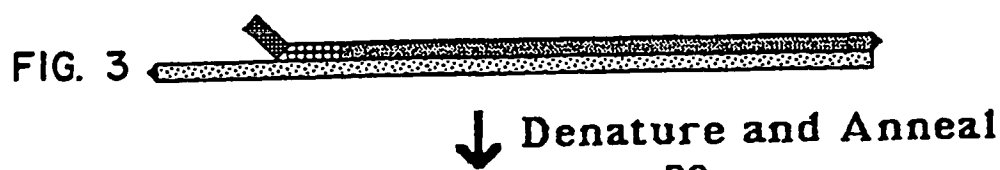
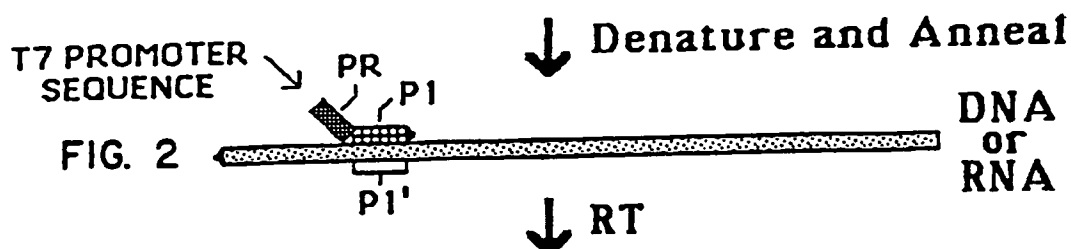
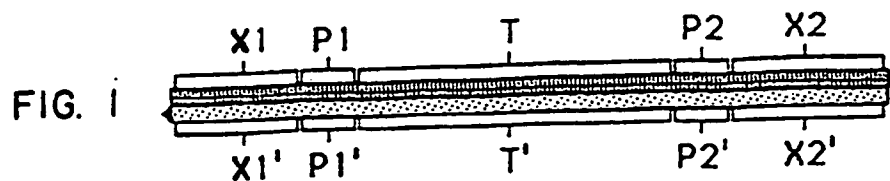
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SUBSTITUTE SHEET



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02601

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC INT CL (4) C12Q 1/68 U.S. CL: 435/6														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border: 1px solid black;">Classification System</th> <th style="border: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: middle;">U.S.</td> <td style="border: 1px solid black;">435/6, 803, 91 536/27; 935/78 436/501, 63</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	U.S.	435/6, 803, 91 536/27; 935/78 436/501, 63								
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Computer Searches: APS; Chemical Abstracts; BIOSIS, Derwent (WPI, WPIL, Biotechnology)														
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border: 1px solid black;">Category <sup>9</sup></th> <th style="border: 1px solid black;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 15%; border: 1px solid black;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">US, A, 4,683,202 (MULLIS) 28 July 1987 see column 13, lines 27-41.</td> <td style="text-align: center; vertical-align: top;">1-15</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">US, A, 4,683,195 (MULLIS ET AL.) 28 July 1987, see column 16, lines 58-68; column 17, lines 1-4 and 15-63.</td> <td style="text-align: center; vertical-align: top;">1-15</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">Biochemistry, Volume 24, issued 08 October 1985 (Washington, D.C.) U.S.A.), Axelrod, V.D. et al "Transcription from Bacteriophage T7 and SP6 RNA Polymerase Promoters in the Presence of 3'-Deoxyribonucleoside 5'-Triphosphate Chain Terminators", see abstract page 5716.</td> <td style="text-align: center; vertical-align: top;">1-15</td> </tr> </table>			Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	Y	US, A, 4,683,202 (MULLIS) 28 July 1987 see column 13, lines 27-41.	1-15	Y	US, A, 4,683,195 (MULLIS ET AL.) 28 July 1987, see column 16, lines 58-68; column 17, lines 1-4 and 15-63.	1-15	Y	Biochemistry, Volume 24, issued 08 October 1985 (Washington, D.C.) U.S.A.), Axelrod, V.D. et al "Transcription from Bacteriophage T7 and SP6 RNA Polymerase Promoters in the Presence of 3'-Deoxyribonucleoside 5'-Triphosphate Chain Terminators", see abstract page 5716.	1-15
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search  <b>17 October 1988</b> </td> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of Mailing of this International Search Report  <b>01 DEC 1988</b> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">           International Searching Authority  <b>ISA/US</b> </td> <td style="border: 1px solid black; padding: 5px;">           Signature of Authorized Officer    <b>JEREMY M. JAY</b> </td> </tr> </table>			Date of the Actual Completion of the International Search <b>17 October 1988</b>	Date of Mailing of this International Search Report <b>01 DEC 1988</b>	International Searching Authority <b>ISA/US</b>	Signature of Authorized Officer  <b>JEREMY M. JAY</b>								
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